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# Fluorescence lifetime imaging ophthalmoscopy adds the retina to cortical pathology for visual dysfunction in neurodegenerative diseases

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Because neurodegenerative diseases such as Parkinson's and Alzheimer's Diseases [AD and PD] as well as the progressive forms of multiple sclerosis [MS] are invariably associated with clinically significant cortical symptoms such as language difficulties, motor skill deficits and cognitive impairments, especially memory, a tacit assumption evolved that visual disorders related to cortical dysfunction must localize only to the temporal, parietal and occipital lobes. Based upon our current understanding, retinal changes in MS are most likely secondary to optic neuropathy, whereas in AD and PD, they appear to represent primary retinal changes. The paradigm was reinforced by the lack of retinal findings using ophthalmoscopy. Spectral domain optical coherence tomography [OCT], optical coherence angiography [OCT-A], and fundus autofluorescence [FAF] have challenged this creed by uncovering structural changes within the retina over and above what can occur as a consequence of optic neuropathy in the case of MS. Still, definitive diagnostic and prognostic data have yet to emerge. Fluorescence lifetime imaging ophthalmoscopy [FLIO], a non-invasive, non-contact, painless imaging technology, measures nanosecond lifetimes of endogenous retinal fluorophores, some of which are linked to mitochondrial activity. Therefore, FLIO is a metabolic, not a structural imaging modality. Because mitochondrial dysfunction occurs in many neurodegenerative diseases, FLIO offers a unique strategy for investigating retinal metabolism in AD, PD, and MS. This article reviews the basic biomedical engineering of FLIO and reports preliminary data from these diseases, correlated with disease duration. These functional *in vivo* data are consistent with retinal metabolic changes in AD, PD, and progressive MS that were "hiding in plain sight" from structural examinations.

## KEYWORDS

fluorescence lifetime imaging ophthalmoscopy, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, multiple sclerosis

## Introduction

Certainly, in its earliest clinical stages and sometimes with more advanced neurodegenerative diseases, cortical visual dysfunction remains an elusive diagnosis. Despite precise localization through advanced magnetic resonance imaging [MRI] technologies (1–4), patients' presenting symptoms are quite imprecise, simulating common ophthalmic disorders

including refractive errors, keratitis sicca, cataracts, glaucoma, and maculopathies including macular degeneration and diabetic retinopathy.

In comparison to acute cerebral infarctions, both common and rare neurodegenerative conditions are associated with non-specific, non-diagnostic MRI findings of cortical atrophy often reported as “age-related” (1–5). Moreover, recent questions have emerged asking if the cortical dysfunction correlates with all the patients’ visual symptoms, especially decreased low contrast visual acuity.

Localizing and diagnosing the cause of visual symptoms can be challenging when the optic nerves and retinas appear normal on direct and indirect ophthalmoscopy, as well as on intravenous fluorescein angiography. However, a normal fundus does not exclude significant retinal and optic nerve dysfunction.

Spectral domain optical coherence tomography [SD-OCT] has generated many reports of abnormalities in both Alzheimer’s and Parkinson’s Diseases (6–19), but no definitive, easily identified, retinal structural imaging biomarkers have emerged to establish an authoritative diagnosis prior to clinically obvious dementia or impaired motor function. Promises for an earlier, more straightforward diagnosis are emerging with both blood and cerebrospinal fluid assays for abnormal proteins (20–26), but costs are a deterrent to widespread evaluation of large numbers of patients. Positron emission tomography [PET] scanning does offer definitive results (23, 27–30), however, accessibility is often restricted due to cost, insurance coverage limitations, and availability, so most patients continue to receive diagnoses primarily based on cognitive testing.

The era of metabolic imaging of the retina using fluorescence lifetime imaging ophthalmoscopy [FLIO] is developing rapidly. Although not yet widely available, FLIO performs non-invasive, painless, rapid imaging to produce both qualitative and quantitative data about the metabolic health of the retina (31–40). FLIO offers insights into visual symptoms and holds promise as potential biomarkers for earlier diagnosis and disease monitoring, even before clinical symptoms appear (34, 35, 37, 38). Since the brain and retina are linked *in utero* by a common neuro-ectodermal origin, specifically the diencephalon, a valid rationale emerges that pathological processes in the brain may also involve the retina (10, 41–44).

Because more and more data are emerging about FLIO and neurodegenerative diseases (35, 45–48), this article will review the technology behind FLIO and report longitudinal data with FLIO correlated with disease duration in relapsing–remitting and secondary progressive multiple sclerosis, Parkinson’s, and Alzheimer’s Diseases.

## Patient report of the effect of Alzheimer’s disease on visual function: cortical or retinal or both?

Born in South Philadelphia in 1933, William Utermohlen, an artist, created self-portraits each year after his diagnosis of Alzheimer’s Disease until he was no longer able to draw (49). His serial drawings in Figure 1 reveal a constellation of visual disturbances, some of which are certainly cortical in origin, with distortion of perspective and a lack of stereopsis. However, some details of his face disappear over the years in a pattern that may be consistent with central and paracentral scotomas but not resembling a homonymous hemianopia. These scotomas possibly localize to the maculae, plus his portraits

reveal loss of some color perception, a finding that may localize to macular cone dysfunction as well as cortical involvement with the perception of color. Mr. Utermohlen died in London in 2007, no longer being able to draw his self-portrait for the last 6 years of his life (50).

In standard diagrams of the regions of the brain affected by Alzheimer’s, the retinas are never included, likely because of the lack of ophthalmoscopic findings in the retina and optic nerve. However, not seeing a process in the ophthalmoscope does not exclude pathological changes below the level of resolution of the ophthalmoscope.

Figure 2 provides precise neurological localization for pathological changes for the “what is it” symptom [ventral pathways] and the “where/how” symptoms [dorsal pathways], both originating from the occipital lobes. But do these areas of the afferent visual system explain all of the abnormalities in Mr. Utermohlen’s portraits and other patients suffering from Alzheimer’s Disease?

## Retinal structural findings in Alzheimer’s disease

In recent years, clinicians and clinical researchers are reporting more and more retinal structural abnormalities in patients with Alzheimer’s Disease (AD).

The retinal findings in patients with Alzheimer’s Disease follows:

1. Amyloid Beta (A $\beta$ ) Deposition (7, 12, 13, 17, 18, 35, 51–57):
  - a. A $\beta$  plaques, a hallmark of AD in the brain, are also found in the retina of AD patients.
  - b. These deposits are often observed in the ganglion cell layer (GCL), inner nuclear layer (INL), and inner plexiform layer (IPL).
  - c. The presence of retinal A $\beta$  deposits can correlate with brain A $\beta$  accumulation and disease severity.
2. Retinal Nerve Fiber Layer (RNFL) Thinning (6–10, 12, 55–57):
  - a. The RNFL, composed of axons of retinal ganglion cells, shows thinning in AD patients, particularly in the superior and inferior quadrants.
  - b. This thinning suggests loss of retinal ganglion cells and their axons, which may contribute to abnormal visual information to the temporal, parietal, and occipital lobes.
3. Ganglion Cell Layer (GCL) Thinning (10–13, 55–57):
  - a. The GCL is also observed to be thinner in AD patients.
  - a. This thinning reflects the loss of retinal ganglion cells, which are crucial for transmitting visual information to the brain.
4. Microglial Activation (53, 58–60):
  - a. Microglia, the immune cells of the central nervous system, are activated in the retinas of AD patients.
  - a. Activated microglia can contribute to neuroinflammation and potentially exacerbate neuronal damage.
5. Vascular Changes (13–19, 61):
  - a. Alterations in the retinal vasculature, including reduced vessel density and increased foveal avascular zone (FAZ), have been reported in AD patients.
  - a. These vascular changes may reflect similar changes occurring in the brain’s microvasculature.
  - a. Dr. Fekrat and Dilraj S. Grewal, MD, also at Duke University, recently reported on OCT and OCTA findings

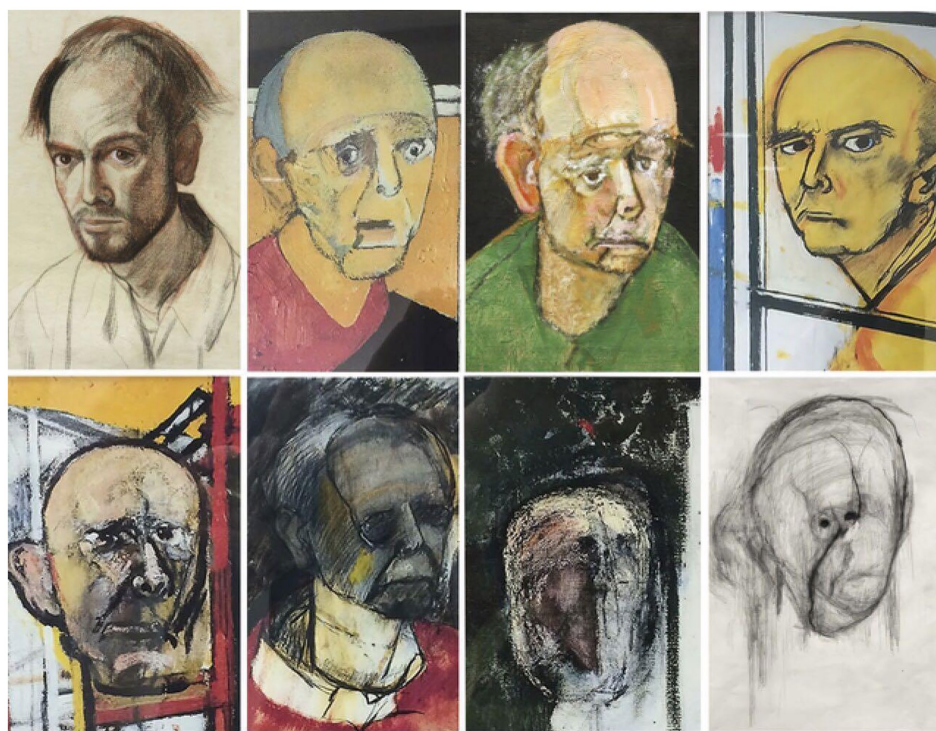


FIGURE 1

After being diagnosed with Alzheimer's Disease, artist William Utermohlen, at the urging of his nurse, created a self-portrait each year until he was no longer able to draw. The first portrait on the upper left was drawn in 1995, and the last on the lower right was in 2001. He died in 2007. Adapted from Crutch et al. (49).

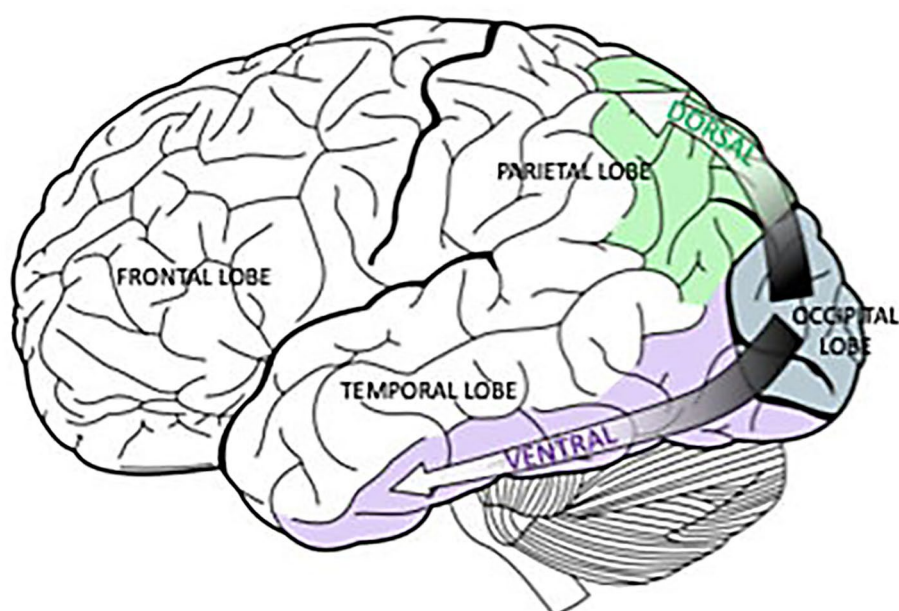


FIGURE 2

Originating in the occipital lobes, the green, dorsal pathway goes to the parietal lobe and is associated with spatial processing. In a similar way, the purple, ventral pathway connects the occipital lobes and the temporal lobe and is linked to object recognition. Selket/Wikimedia Commons, GNU Free Documentation License, CC BY-SA 3.0. <https://solportal.ibe-unesco.org/articles/building-a-brain-that-can-read-part-1-sound-and-sight/>.



in the superficial capillary plexus in eyes of patients with mild cognitive impairment (MCI) and AD (16). They found reduced macular vessel density, perfusion density, and ganglion cell-inner plexiform layer thickness in eyes with AD compared to those with MCI and normal controls.

6. Melanopsin-containing Retinal Ganglion Cell (mRGC) Loss (62–64):
  - o A specific subpopulation of retinal ganglion cells, the mRGCs, appear to be vulnerable in AD.
  - o These cells play a role in regulating circadian rhythms, and their loss may contribute to sleep disturbances and circadian dysfunction observed in AD.
7. Tau Pathology (8, 54, 65–67):
  - o Hyperphosphorylated tau, another hallmark of AD, has also been found in the retina of AD patients.
  - o The role and distribution of tau pathology in the retina, as well as its relationship to Amyloid- $\beta$  ( $A\beta$ ) accumulation, have been identified in the retina for neurodegeneration-associated disorders like Alzheimer's disease (AD) and glaucoma.

## FLIO—metabolic imaging of the retina and the principles of fluorescence lifetimes

Over the past decade, university researchers have collaborated with industry to prototype and refine methods for assessing the metabolic environment of the retina, one of which is fluorescence lifetime imaging ophthalmoscopy [FLIO]. FLIO is the *in vivo*, clinical application of the well-validated research technology called fluorescence lifetime imaging microscopy [FLIM]. While most of the currently published FLIO data have been generated to date using the Heidelberg Engineering Spectralis-based device to measure fluorescence lifetimes, other groups have developed different *in vivo* retinal imaging systems to measure metabolic activity (36, 40, 68–73). The growing interest in retinal metabolic imaging will add a functional dimension of imaging and understanding of disease to established structural imaging modalities in a clinical setting.

The *in vivo* metabolic imaging method of FLIO complements high-resolution optical coherence tomography in a similar way that positron emission tomography [PET] scanning uses radioactive tracers to evaluate tissue function, thereby adding metabolic data to magnetic resonance imaging [MRI] scanning. However, compared to PET scanning, FLIO leverages the endogenous fluorophores of the retina to make the previously invisible metabolic functions visible. Therefore, FLIO fulfills many criteria for an ideal imaging biomarker because it is non-invasive, non-contact, painless, and performed quickly, producing data that is immediately available for analysis, and without requiring radioactive materials or imaging contrast agents. Dilation of the pupils is the only “invasive” part of FLIO.

## Fluorescence lifetimes

G. G. Stokes, the Lucasian Professor of Mathematics at Cambridge University, where he spent his entire career, including graduation from Pembroke College, discovered the property of fluorescence, the ability of certain materials to convert invisible ultraviolet light into light of longer wavelengths within the visible spectrum (74–76). FLIM and

FLIO apply Stokes' magnificent discovery made in 1852 to the real world of medicine in the 21st century.

From Stokes' elegant work, the concepts of fluorescence intensity and fluorescence lifetimes were born. Fluorescence intensity refers to the brightness of fluorescence or the amount of light emitted by a molecule or compound when it is excited by another light. Greater intensity usually means a greater concentration of a molecule or a fluorescently labeled target. Fluorescence intensity is one of the principles of flow cytometry. However, intensity measurements are subject to a variety of artifacts, including uneven illumination and photobleaching, as well as motion and optical aberrations (74–76).

In contrast, fluorescence lifetime imaging avoids many of the previously mentioned artifacts and records the average time a molecule spends in the excited state before returning to the ground state. These lifetimes are in the nanosecond range ( $10^{-9}$  s). Every molecule has a unique fluorescence lifetime signature so that individual molecules can be detected at different time points in a physiological or pathophysiological process. The current FLIO setup, which is only for investigational, not medical, care, purposes, is shown in Figure 3. In this system, the retinal fluorophores are excited by a 473-nm pulsed laser and the emitted fluorescence is simultaneously detected in two distinct wavelength channels: a short spectral channel (498–560 nm) and a long spectral channel (560–720 nm). Table 1 from Professor Christine Curcio's research laboratory at the University of Alabama Birmingham Heersink School of Medicine clearly describes the compounds imaged in the short and long wavelength channels (77). The current FLIO version does not record the fluorescence signature of individual molecules; however, future generations of FLIO may use two-photon imaging, enabling detection of specific fluorophores involved in normal and abnormal mitochondrial, endoplasmic reticulum, and lysosomal functions (77).

## Normal FLIO and proof of concept with macular telangiectasia type 2

Figure 4 illustrates the normal FLIO images for the short and long spectral channels performed in a healthy control (45). FLIO data must be analyzed with control data for both gender and age, and possibly for ethnicity.

Professor Paul Bernstein and his team at the University of Utah have pioneered FLIO for macular telangiectasia type 2, a rare disease, to establish this technology as an imaging biomarker whose diagnostic and longitudinal signals far exceed fundus photography, intravenous fluorescein angiography, fundus autofluorescence, and optical coherence tomography. The data from Professor Bernstein's group is illustrated in Figure 5 (34, 37, 38).

## Analyzing FLIO data from Parkinson's and Alzheimer's diseases and multiple sclerosis

Because FLIO captures fluorescence lifetimes in a 256 by 256-pixel area covering a 9 by 9 mm area ( $35\ \mu\text{m} \times 35\ \mu\text{m}/\text{pixel}$ ) of the retina, targeting the macula or other areas of the retina, analyzing FLIO data

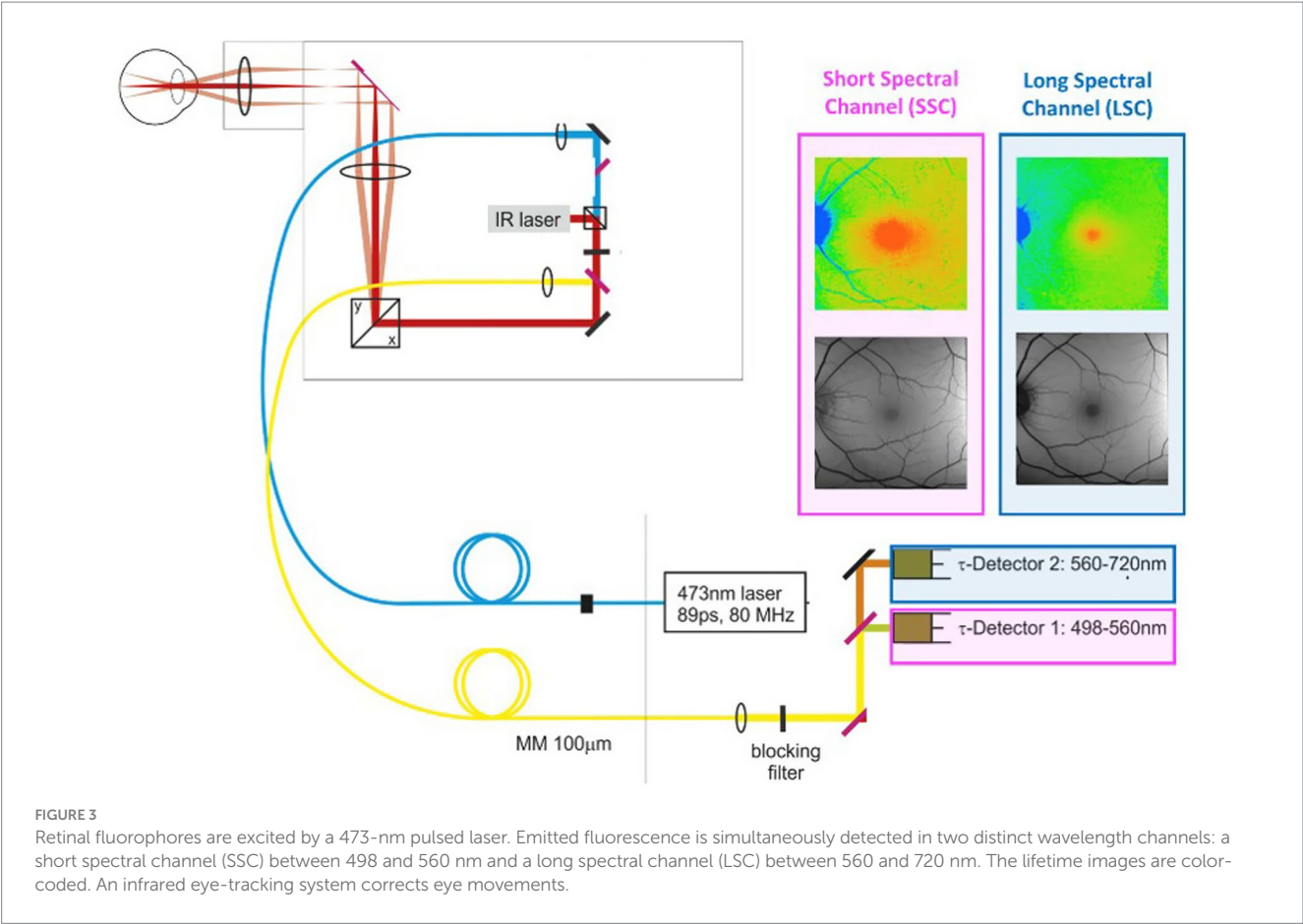


TABLE 1 Retinal signal sources for fluorescence lifetime imaging ophthalmoscopy.

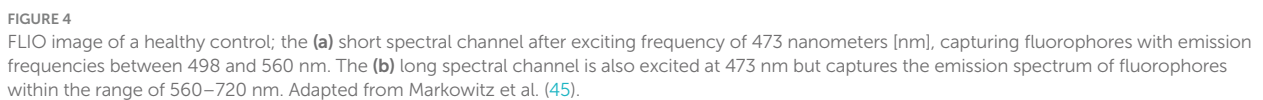
| Fluorophore                                        | Cell/Compartment                                                                                                                                                                                                        | Captured by |
|----------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| Tryptophane                                        | Membranes of photoreceptors, amacrine cells, and bipolar cells                                                                                                                                                          | LSC/SSC     |
| Nicotinamide adenine dinucleotide (NADH, NADPH)    | Mitochondria; in particular in the ellipsoid zone, basal RPE, and OPL                                                                                                                                                   | LSC/SSC     |
| FAD                                                | Mitochondria; in particular in the ellipsoid zone, basal RPE, and OPL                                                                                                                                                   | LSC/SSC     |
| Melanin                                            | RPE melanosomes, melanopolipofuscin, and choroidal melanocytes                                                                                                                                                          | LSC/SSC     |
| Bisretinoid derivatives of vitamin A intermediates | Lysosome-related organelles (lipofuscin and melanopolipofuscin) in RPE                                                                                                                                                  | LSC > SSC   |
| Collagen, elastin                                  | Internal limiting membrane, vitreous cortex, vessels, retinal nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, vessels, Bruch's membrane | LSC/SSC     |
| Macular xanthophyll carotenoids                    | Membranes of Müller glia, photoreceptors, RPE                                                                                                                                                                           | SSC         |

Adapted from Goerdts et al. (77).

is quite complex and still a work in progress. SPCImage (Becker & Hickl GmbH) is the most commonly used software for analyzing FLIO data currently. This software uses a triexponential approach to fit fluorescence decay curves at each pixel because each pixel has thousands of datum points, one data point for each time point (31–33, 39, 78, 79). The triexponential approach fits all data points into three lifetime components, each with its respective amplitude, reflecting the percent contribution of the fluorescence lifetimes in the fit model. The final output of the triexponential decay model is plotted with a

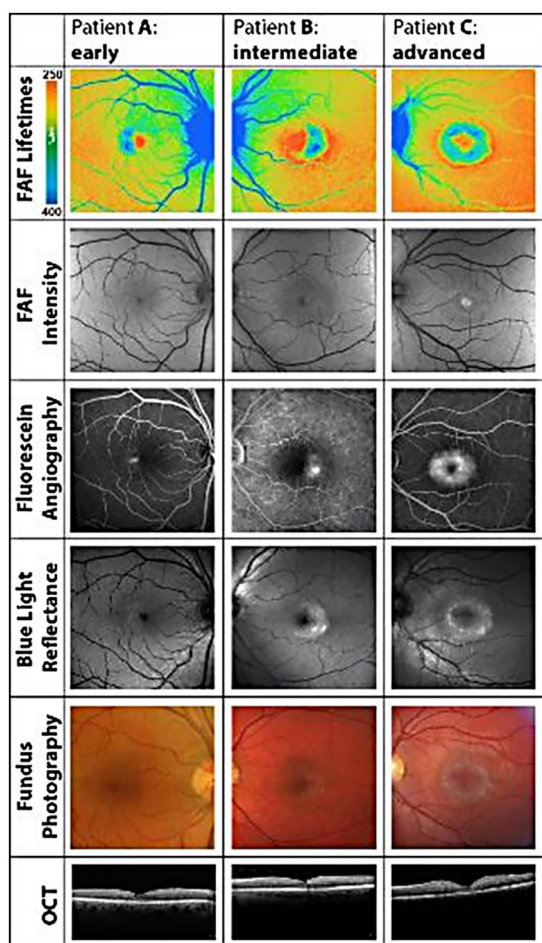
color-coded heat map, representing the mean fluorescence lifetime at each pixel. This process produces colorful images of the area of the retina, where one can quickly identify areas of shorter or longer fluorescence lifetimes, providing qualitative information about the metabolic function in different regions. Each pixel from the fit model in SPCImage displays the mean fluorescence lifetime, which is useful for further quantitative analysis.

While SPCImage is the software used for calculating and fitting mean fluorescence lifetimes, post-processing analysis is helpful for



post-process the fluorescence lifetime data. These programs provide statistical analyses of lifetimes across and within groups of eyes. Most importantly, these programs can apply a standardized region of interest (ROI) to the FLIO images, such as the Early Treatment





**FIGURE 5**  
Illustrates how FLIO detects pathological changes in macular telangiectasia type 2 before any of the other retinal imaging modalities. Please compare the FLIO images in this figure to the normal FLIO images for the short spectral channel in [Figure 4](#). Adapted from Sauer et al. (34).

Diabetic Retinopathy Study (ETDRS) grid. After overlaying an ROI, such as the ETDRS grid, to each FLIO image, it is possible to quantify the means of individual lifetime components within each of the pixels that fall in a specific zone of the grid. This post-processing also allows for combining multiple areas of the ETDRS grid to identify quantitative regional fluorescence lifetime patterns from specifically defined ETDRS zones. This process, along with additional analyses, creates a more standardized and detailed approach to analyzing fluorescence lifetime data across patient eyes in various disease conditions.

## Parkinson's disease

Regardless of whether Parkinson's develops from a variety of genetic mutations or environmental toxic exposures, such as MPTP, rotenone, and paraquat, or sporadically, the final common pathway involves dysfunction in mitochondrial ATP production, impaired

mitophagy, and abnormal protein aggregation (80–84). Since one-photon FLIO captures a combination of signals that emanate in large part from FAD, NAD, and NADH, endogenous fluorophores known to be associated with oxidative phosphorylation and mitochondrial generation of ATP, we studied a cohort of patients with Parkinson's Disease stratified by time from the initial diagnosis. [Figure 6](#) illustrates FLIO changes in two Parkinson's disease patients, one 12 years after the time of diagnosis and the other 18 years after the time of diagnosis (45, 46). FLIO data in Parkinson's Disease demonstrates prolonged fluorescence lifetimes in both the short and long spectral channels (46, 47).

## Alzheimer's disease

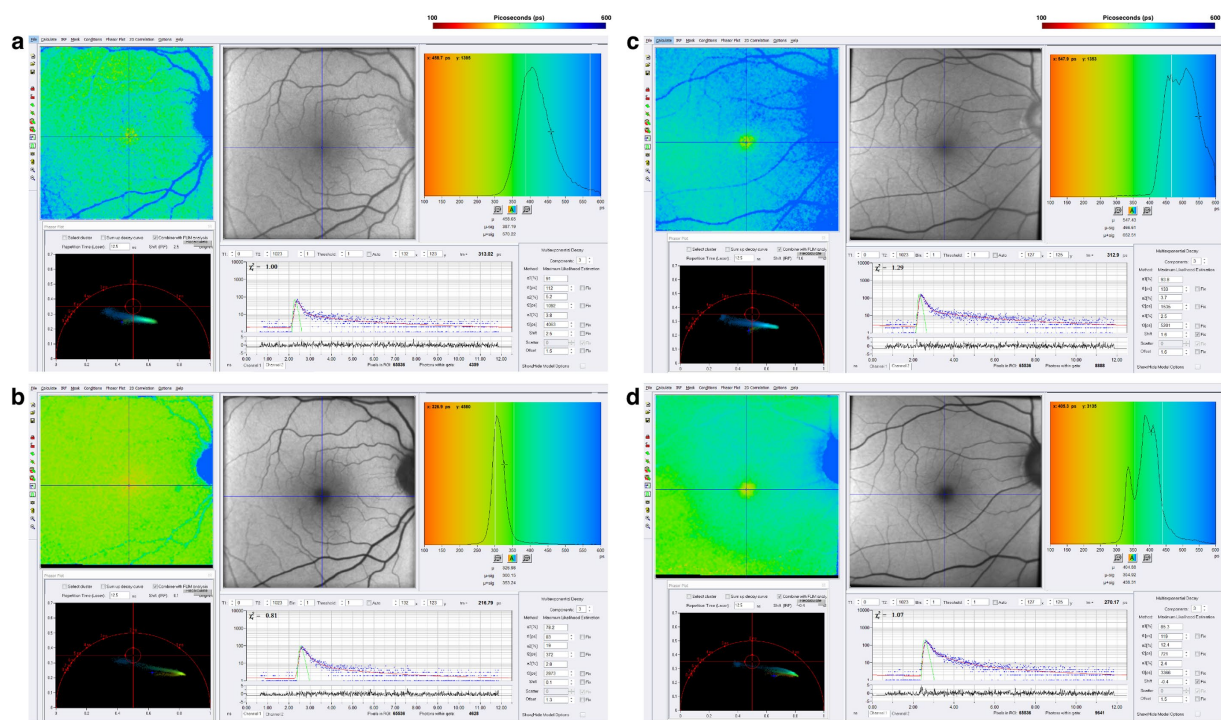
One-photon FLIO data in Alzheimer's Disease demonstrates prolonged fluorescence lifetimes in both the short and long spectral channels, as seen in the comparison of Alzheimer's disease patients with two- and four-year histories of the disease ([Figure 7](#)). Two-photon FLIO, which is now under consideration for development, may be able to determine the molecular basis for these differences in the retinal microenvironment. More and more data implicate mitochondrial dysfunction as the possible origin of Alzheimer's Disease (85–87). Specifically, the “Mitochondrial Cascade Hypothesis” postulates that abnormal mitochondrial function impacts the processing of amyloid precursor protein (APP), a transmembrane protein, and increases A $\beta$  oligomer production and accumulation, possibly promoting the formation of neurofibrillary tangles (88).

## Multiple sclerosis – progression independent of relapse activity “smoldering MS”

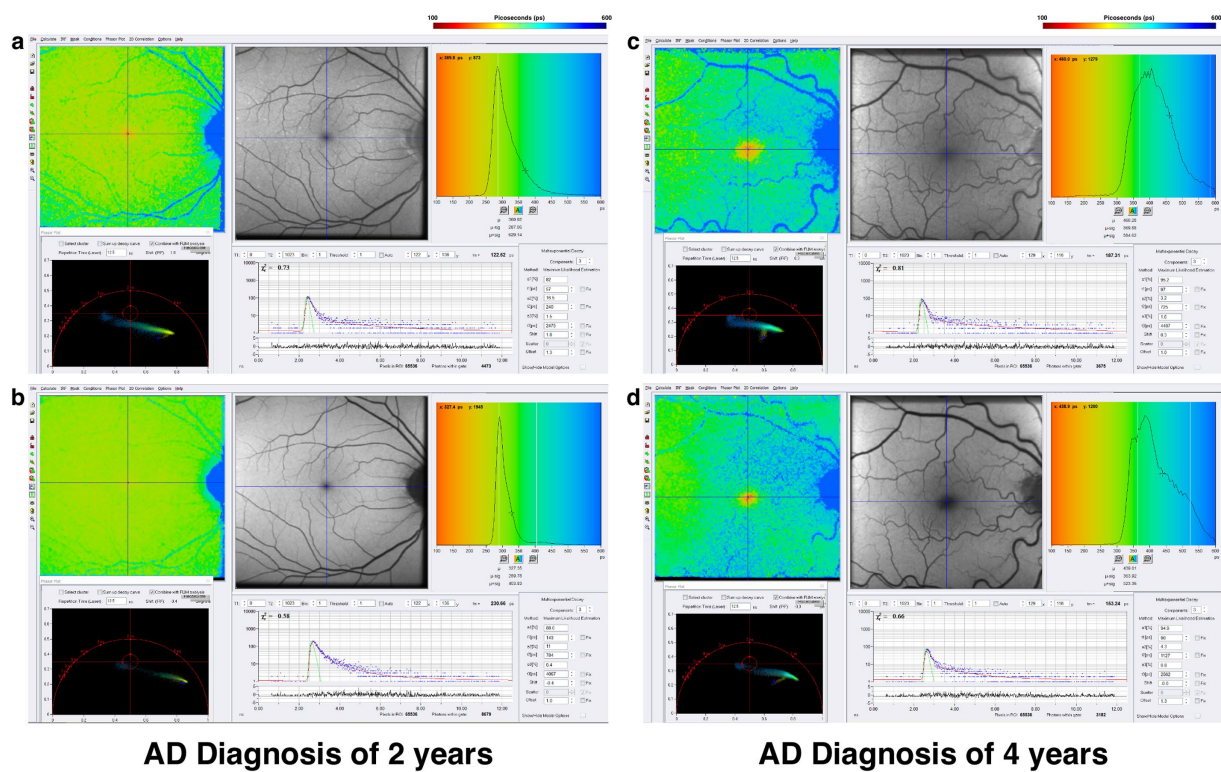
During the past 4 years, the concept and validation of progression independent of relapse activity [PIRA] or “smoldering MS” has received increasing attention in both multiple sclerosis treatment and research (89–94). PIRA asks and answers the important questions about increasing disability and transition from relapsing–remitting MS [RRMS] to secondary progressive MS [SPMS] and what serological or imaging biomarkers might predict this transition. PIRA or smoldering MS represents a distinct, disabling component of this disease, not addressed by the currently available MS medications designed to reduce clinical relapses and MRI disease activity.

Patients are classified as PIRA when they experience progressive disability without experiencing any clinical relapses, or new inflammatory lesions with MRI scanning.

Chronic neuroinflammation, which is below the resolution of MRI scanning, has been suggested as a pathophysiological mechanism for PIRA (90–94). Data from our group presented at the 2025 Americas Committee for Treatment and Research in Multiple Sclerosis [ACTRIMS] annual meeting suggests that mitochondrial dysfunction may also be involved (48). [Figure 8](#) illustrates FLIO changes of a patient with RRMS and SPMS (48). FLIO data in both MS patients demonstrates prolonged fluorescence lifetimes mainly in the short spectral channel.



**FIGURE 6**  
FLIO images of a patient with a 12-year diagnosis of PD showing both the (a) SSC and (b) LSC. Also shown is a patient with an 18-year diagnosis of PD showing both the (c) SSC and (d) LSC. Adapted from Markowitz et al. (45), and Shivok et al. (46).



**FIGURE 7**  
FLIO images of a patient with a 2-year diagnosis of AD showing both the (a) SSC and (b) LSC. Also shown is a patient with a 4-year diagnosis of AD showing both the (c) SSC and (d) LSC. Adapted from Markowitz et al. (45).



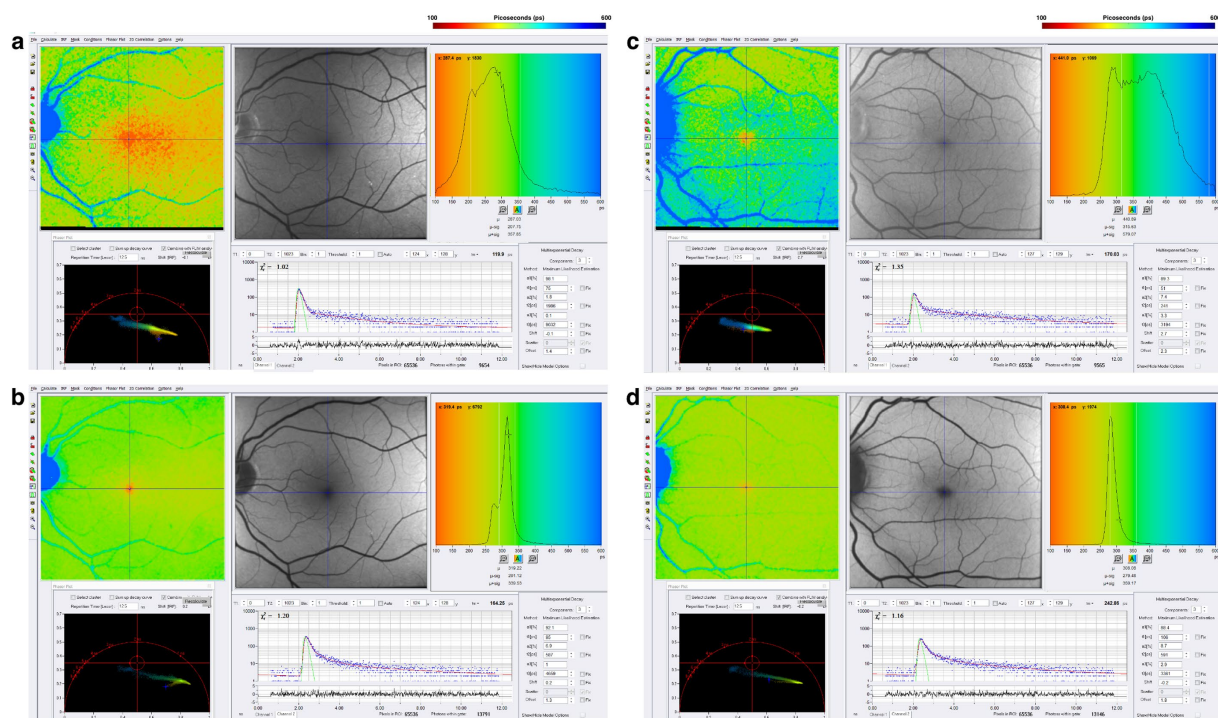


FIGURE 8

FLIO images of a patient with RRMS and 8 years of disease duration showing both the (a) SSC and (b) LSC. Also shown is a patient with SPMS and 38 years of disease duration, showing both the (c) SSC and (d) LSC. Adapted from Markowitz et al. (46).

## Conclusion— FLIO insights and two-photon future

FLIO adds the new imaging, biomarker dimension of retinal metabolism and mitochondrial function to the existing arsenal of MRI, PET, and OCT scanning, to investigate the origins of punishing neurodegenerative syndromes, as well as a method to monitor a previously unseen disease activity.

The current one-photon FLIO setup may transition to a two-photon setup pending complete safety evaluations. Therefore, FLIO will follow the same course of MRI, CT, PET, and OCT with the images and data improving as physicists, biomedical engineers and clinical researchers unite to develop two-photon FLIO.

We anticipate that FLIO will earn an important position in the following dimensions of neurodegenerative diseases, especially when merged powerful *in vitro* methods of focused ion beam scanning electron microscopy [FIB-SEM, 3-dimensional images with 3 nanometer resolution] and fluorescence lifetime imaging microscopy [FLIM] studying retinoids and brain organoids derived from inducible pluripotent stem cells.

We foresee the following meaningful FLIO advances for patients and their families: [1] enabling an early diagnosis of disease activity especially in genetically at-risk populations or patients with early stage tremor for Parkinson's Disease and patients with mild cognitive impairment [MCI] for Alzheimer's Disease; [2] following the clinical course of the diseases with mitochondrial fluorophores and fluorophores of other pathologically important molecules for an early imaging biomarker of disease progression for many neurodegenerative diseases before

worsening clinical symptoms and signs; and [3] discovering and evaluating in clinical trials molecules that specifically target mitochondrial dysfunction; thereby, addressing the metabolic abnormalities revealed by FLIO, as the process of “making the invisible visible” produces real-world benefits for patients, their caregivers, and their families.

## Limitations

FLIO currently faces several limitations. High cost and limited accessibility restrict its use, with fewer than 30 centers worldwide operating FLIO systems. Standardization across centers is needed, as current measurements and results cannot be directly compared between sites. Greater understanding of FLIO results, including test-retest variability, is required. The current one-photon FLIO setup does not resolve fluorescence signatures of individual molecules; however, future two-photon versions may enable detection of specific fluorophores involved in mitochondrial, endoplasmic reticulum, and lysosomal functions.

## Future directions

Future studies should be designed to observe FLIO changes longitudinally across disease stages, duration and subtypes, using larger patient cohorts and healthy controls stratified by age and sex. Developing disease-specific and healthy control reference databases, combined with AI-based analysis, will improve automated,

reproducible detection of disease-related fluorescence abnormalities and enhance FLIO's clinical utility.

## Author contributions

JN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. DM: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Software. RS: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## Conflict of interest

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